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Breast cancer presumably originates in an estrogen receptor-positive (ER+), estrogen (E2)-responsive epithelial cell within organized human breast tissue. It is a paradox that there is an exceedingly low level of, and few cells expressing, ER in normal breast tissue, and human breast epithelial cell lines (HBE-CL) lack ER and do not respond to E2. Our hypothesis is that human breast parenchymal epithelial cell-cell interactions and epithelial stromal interactions are critical *in vitro* determinants of HBE cell ER expression and biochemical differentiation. We have made substantial progress, in spite of staff changes, in the portion of Specific Aim 1 proposed for year one, including development of required new mortal cell reagents, e.g. fibroblasts from reduction mammoplasty, benign breast and breast tumor tissues, as well as new immortalized cell lines. We have formed the unique conglomerate structures that resemble human breast parenchymal organization *in vivo*, and have begun evaluation for ER expression and E2 growth responsiveness. The negative results to date are not unexpected, they substantiate the paradox, and emphasize the importance of accomplishing our long term goal-recapitulating *in vivo* organization *in vitro* to evaluate it's potential to bring about ER expression and/or E2 growth responsiveness.

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FOREWORD

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Robert J. Pausley   6/17/98
PI - Signature   Date

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(5)   INTRODUCTION:

Subject: This research project's subject is to examine cellular organization, focusing upon epithelial-epithelial and epithelial-stromal interactions, and environment as potential determinants of both estrogen receptor expression in, and differentiation by, human breast epithelial cells.

Purpose: The rationale for this proposal was that it is generally assumed that breast cancer originates in estrogen receptor-positive [ER+], estrogen [E2]-responsive epithelial cells within structurally organized human breast tissue. Although this assumption is probably valid it is a paradox that there is an exceedingly low level of, and few human breast epithelial cells [HBE] expressing ER in normal breast tissue; a paradox extending to human breast epithelial cell lines [HBE-CL] that lack ER and do not respond to E2. The hypothesis this proposal tests is that human breast parenchymal epithelial cell-cell interactions, and epithelial stromal interactions including epithelial-ECM and stromal fibroblast interactions, are critical determinants of ER expression and biochemical differentiation in HBE cells and cell lines *in vitro*. Further, acting in this organized context the environment of hormones and growth factors are additional critical determinants of ER expression and differentiation.

Scope of Research: The scope of this research is described in the experimental plan and specific aims. The experimental plan testing this hypothesis uses mortal [short-term cultures of] HBE cells and some of the MCF10 family of HBE-CL cultured in monolayer, in collagen to foster organization, in Matrigel to simulate the ECM, and in three-dimensional structures [formed in response to a shift from a low to routine Ca^{++} environment] resembling breast parenchymal organization *in vivo*. Breast stromal fibroblasts will be added to each condition because of evidence that these cells facilitate preservation of ER expression in rodent mammary epithelial cells *in vitro*.
     The experimental plan has three specific aims. First, HBE and HBE-CL are to be cultured under these conditions to determine if interactions between epithelial cells, with ECM, and/or with stromal fibroblasts preserves ER expression in primary cultures and/or restores ER expression in HBE-CL, as well as functional response [increased PgR expression and cell proliferation] to E2. Second, analysis of these interactions will be undertaken to characterize them microscopically, and for the expression of molecular determinants, including cadherins and laminins, that mediate these interactions. Third, the functional status of these interactions will be examined by testing the ability of these cells to undergo biochemical differentiation, β-casein and α-lactalbumin expression, in response to their organizational status and environment of hormones and growth factors.

Background of previous work: This background of previous work primarily focuses upon research pertaining to, and describes our experience with, the cell reagents required for this proposal.
     A major focus in breast cancer research has been establishment and characterization of human breast epithelial cell lines [HBE-CL] that are "normal" in terms of not being tumorigenic; we recognize no cell line, by definition, can be "normal". The target was the breast parenchymal ductal-luminal cell, not parenchymal myoepithelial or stromal cells, because by several criteria these are the cells in which breast cancer originates. This goal has been accomplished to some, but not yet sufficient extent; e.g., our establishment of MCF-10A and other HBE-CL (1-4) along with cell line models of breast cancer development; e.g., MCF10 derivatives (4-10). All had probably expected that HBE-CL would express the estrogen receptor [ER] but invariably they lacked ER (1,3,11).
     The method to propagate human breast epithelial cells from subcutaneous mastectomy and reduction mammoplasty tissues has these major features: culture in a low [0.04-0.06mM] Ca^{++} medium, passage of viable free-floating cells released from confluent

monolayer cells, and non-serial passage to permit long term propagation (1,12,13). Mortal cells, using guidelines for phenotyping human breast cells (14-19), are of breast ductal/luminal epithelial origin based upon expression of cytokeratins [Ck]18 and 19 and of breast polymorphic epithelial mucin/EMA-epithelial membrane antigen/HMFG-human milk fat globule antigen/sialomucin/episialin, in contrast to myoepithelial or stromal cells. Mortal cells have a human diploid karyotype (1,20).

Mortal MCF-10M and MCF-10MS cells from one individual's subcutaneous mastectomy tissue [SCM-130] have on at least four occasions spontaneously immortalized. The MCF-10, MCF10-2, MCF10-3 and MCF10-4 cell lines do not undergo senescence upon serial passage into standard 1.05mM Ca^{++} medium (1,3,4). There are two sublines F and A of each, grown in low and standard Ca^{++} medium, respectively. The unique karyotype of each line proved that each arose from independent spontaneous immortalizations (1,20). These lines lack detectable estrogen receptor (1,3). All are human breast epithelial cell lines by the criteria noted above, however there are differences. By *in vitro* and *in vivo* criteria these cells are not tumorigenic (1,2,4).

The potential of mortal MCF-10MS and MCF-10 cells lines for organization has been examined to some extent. MCF-10A form three dimensional structures "domes", that are characteristic of rodent mammary epithelial cells in prolonged monolayer culture (1,3) [10A distributed by ATCC before 1998 had lost this capacity]; 10-2A and 10-3A form fewer and no domes, respectively (4). In collagen, 10A produced duct-like outgrowths; formation was hormone dependent, maximum duct formation occurred with hydrocortisone in the absence of EGF and insulin for cells cultured in $\leq 5\%$ equine serum, growth was disorganized in $> 5\%$ equine serum (1). MCF-10A reportedly (21) organizes in Matrigel and produces typeIV-collagen, a component of the basement membrane. Human breast epithelial cells including MCF-10MS, 10-2F and 10-3F lines when transferred from low to standard Ca^{++} medium undergo in a matter of hours aggregation into structures termed "conglomerates", indicating complex branching duct-like structures with end buds resembling acinar-like structures (4). This organization resembles the *in vivo* breast parenchymal morphology of the terminal ductal lobular unit [TDLU] (22) and of organoids produced by collagenase + hyaluronidase digestion of breast tissue used to culture breast cells (12,13).

We also have experience with culture of human breast stromal fibroblast-like cells. *In vivo* these cells have the highest amount of immunohistochemical aromatase (23), a rate-limiting enzyme that produces the estrogen precursor estrone. We examined (24) aromatase expression and regulation in cells cultured up to 9 passages from breast tumors and ipsilateral breast tissue distal to the tumor. These cells were characterized immunocytochemically as cytokeratin negative, α-smooth muscle actin and vimentin positive fibroblasts. Aromatase enzyme activity was increased over 1000-fold by treatment with dexamethasone alone or in combination with dbcAMP and phorbol ester. Aromatase mRNA by RT-PCR increased approximately 30 fold. Increased enzyme activity and mRNA accumulation were dose and time dependent. Therefore, aromatase expression occurs in and can be regulated dramatically in breast fibroblasts.

(6) BODY:

The DOD, at the time the proposal was in the decision process for funding, was informed co-investigator Dr. Herbert D. Soule passed away January 2, 1997. This has impacted this project, nevertheless substantive progress has been accomplished with Mr. Steven Santner joining this effort. The PI collaborated with Mr. Santner on the breast fibroblast aromatase experiments described above, it was Mr. Santner who cultured fibroblasts from breast tumor and ipsilateral benign breast tissue. Fortunately in the year prior to Dr. Soule's death he and Mr. Santner collaborated on the development of a new highly tumorigenic derivative of the MCF10AneoT cells, which is not part of this proposal, as well as in the culture of human breast epithelial cells. The cell culture work we have accomplished

under this proposal has been done by Mr. Santner since he joined the project in October. BAKCI, since January 1997, requires at least 20% salary coverage of the PI from each grant; therefore the PI's effort/salary coverage was administratively increased to 20% by rebudgeting Dr. Soule's salary.

Results and Discussion:

To begin to accomplish Specific Aim #1 required, as stated in the proposal, new cell reagents, specifically "new short term culture [primary-10 passage] reduction mammoplasty cells" ... "stromal cells are short term [1-10 passage] reduction mammoplasty-derived fibroblasts, secondarily tumor-derived fibroblasts". Following we report progress in the prerequisite cell cultures, and on the three-dimensional organization of human breast epithelial cells.

Tissues-Acquisition and Characterization: We have implemented, as part of our study, routine histological characterization of the majority, and recently all, tissue specimens. The justification for this effort is our experience, for example the MCF-12 cell line was from a women undergoing a reduction mammoplasty procedure who in fact had prior contralateral breast cancer-which was realized only during preparation of our publication, and the following observations.
                                                              Seven breast tumor tissue and ipsilateral benign breast tissue samples have been obtained and processed for histology. Confirming histology for tumor tissue is from the Tissue Resources Core, coded and edited to eliminate patient identifiers, which arrives about a month later; for benign breast tissue histopathology is not done, therefore confirming reports are not available. Of three "tumors" with confirmation, one tumor had no identifiable tumor cells. Of the four "tumors" without confirmation, two lacked identifiable tumor cells. Of five benign tissues, four exhibited a normal breast epithelial histology and at least some stromal fibrosis; one benign tissue had CIS/carcinoma in situ, a concern that will be kept in mind as any cells derived might, but probably won't, be used. Therefore, we have two reliable, and two probably reliable, benign breast-breast tumor paired samples to date.
                                                              Five reduction mammoplasty tissue samples have been obtained and processed. Of four, with two confirmed, there is no evidence of abnormal histology such as tumor, CIS, or any form of hyperplasia, but in general a paucity of epithelial cells in the tissue.

Cell Cultures: As stated in the experimental plan short-term cultures of reduction mammoplasty, benign breast and breast tumor epithelial cells and fibroblasts were cell resources required for this proposal. A substantial portion of our effort in this phase of the project has gone to the generation of these cell resources. The experimental methods and procedures for culture of cells are as reported in our publications.

Cells Cultured: Epithelial cells- 3/3 reduction mammoplasty, 2/3 benign breast tissues and 0/4 breast tumors have been propagated. Fibroblast cells- 3/3 reduction mammoplasty, 3/3 benign breast and 4/4 breast tumors have been propagated.

Cell Line Development: We have realized that mortal cells, both epithelial and fibroblast, represent a finite resource for our research, and one that variation in properties between cells cultured from the same types of tissue (reduction mammoplasty, benign breast and breast tumor) may negatively impact variation between experiments. We therefore added to our experimental plan the development of cell lines of both epithelial and fibroblast nature from these three tissue sources. Dr. Robert Bright of our institution provided a retrovirus vector containing human papilloma virus E6 and E7 oncogenes (25) to immortalize cells, which we have learned requires infection of early [<4] passage cells. We define these as immortalized cell lines because proliferation continues beyond the time [about 10 passages] parallel uninfected cells cease propagating or senesce. Progress: at this point we have fibroblast cell lines derived from two matched breast tumors and two benign breast tissues, a fibroblast cell line from one reduction mammoplasty, and epithelial cell lines from two reduction

mammoplasty tissues.

Cell and Cell Line Characterization: Cell morphology is cuboidal for epithelial and elongated for fibroblasts mortal cells and cell lines. Immunocytochemical analyses have been done as proposed. Both mortal cells and cell lines are the same for the expression of epithelial and fibroblast phenotypic markers: Epithelial mortal cells and cell lines are pan cytokeratin and cytokeratin 14 positive whereas Fibroblast mortal cells and cell lines have <5% cytokeratin weakly positive cells. Vimentin is not a distinguishing phenotypic marker between cell types, wheras a portion of fibroblasts express a high level of α-smooth muscle actin. These results indicate that epithelial and fibroblast mortal cell cultures and cell lines have distinguishing phenotypic properties, and that cell lines preserve phenotypic markers of parental mortal cells.

Our original proposal presented data demonstrating the capacity of short-term [mortal] fibroblasts from benign breast and breast tumor tissues to express CYP19/aromatase mRNA and enzyme activity. We have examined this property in fibroblast cell lines derived from benign breast and breast tumor tissue-derived mortal cells, examining both mortal cells and cell lines of the same lineage. Our results demonstrate [data not shown] that CYP19 mRNA is upregulated in mortal cells and cell lines from two benign breast and breast tumor samples. We have extended these same observations to mortal cells from two reduction mammoplasty tissues and are in the process of examining cell lines. The importance of these results are that [1] we have generated fibroblast cell lines from benign breast, breast tumor, and reduction mammoplasty tissue, and [2] that fibroblasts from both mortal cells and cell lines respond to modulators that will be examined in this research. Epithelial mortal cells and cell lines have and are being examined also; to date our results demonstrate that these cells derived from two reduction mammoplasty tissues lack CYP19 mRNA and lack upregulation of mRNA by the same modulators that influence expression in fibroblast cells and cell lines.

Three-Dimensional Organization of Epithelial Cells: Our original proposal included the generation of "conglomerate" structures of certain epithelial cells; termed conglomerates to indicate the complex organization of polarized epithelial cells and lumen-resembling spaces and were described in the Preliminary data. These were MCF10-2F and MCF10-3F cells, routinely cultured in low Ca^{++} medium, that when switched to routine [high] Ca^{++} medium formed complex three-dimensional structures that macroscopically and microscopically resemble the organoids characteristic of breast parenchymal tissue initially out of the host.

We have initiated cell cultures to generate these conglomerates from frozen stocks of cells. Our results to date indicate that prolonged culture [>1 month] is required following reactivation of a frozen stock. We have had to go back to frozen stocks because continuously passaged cell cultures were terminated following Dr. Soule's death. We have succeeded in forming conglomerates from MCF10-2F and MCF10-3F; these are being processed for morphology and immunochemistry. One reduction mammoplasty, placing cells into agar coated multiwells, did not form conglomerates but these mortal cells based upon our experience are exhibiting an exceedingly high growth potential we have not observed before. We have done these experiments with the ER+, E2-responsive human breast cancer cell line MCF7, which will form loose aggregates without obvious morphologic organization, to provide ER+ controls for expression analyses [immunochemistry]. No difference in MCF7's ability for form these loose aggregates has been noted in three experiments comparing cells growing with E2 supplementation as compared to E2 depleted medium.

Estrogen responsiveness and ER expression: We have examined for ER expression in monolayer cultures and for E2 growth responsiveness in monolayer cultures of MCF10 and MCF10-2

cells, as well as MCF7 as a positive control for ER expression and E2 growth responsiveness. No reliable differences in growth rate of MCF-10 or MCF10-2 cells have been noted culturing in E2 supplemented media or in media with an anti-estrogen [Tamoxifen or ICI 182780]. In contrast with MCF-7 cells there was an approximately 4-fold increase in growth with E2 supplementation compared to E2-depleted or anti-estrogen treated cell cultures. Immunocytochemical analyses to date have detected no ER in MCF-10A cells; MCF10-2A cells have less than 5% of cells staining weakly which we do not regard as a reliable postive signal. Our experimental plan this year will be expanded by examining for ER expression by RT-PCR methods, to attempt independent confirmation of possible ER expression in MCF10-2 cells.

(7) CONCLUSIONS:

The Results described above provide evidence of progress in the key elements of Specific Aim 1 which was projected to encompass years one and two of this project, including:

[1]   Generation of mortal human breast epithelial cells from fresh reduction mammoplasty tissue.

[2]   Propogation of fibroblasts from fresh reduction mammoplasty, benign breast, and breast tumor tissues.

[3]   Development of new immortalized cell lines from reduction mammoplasty epithelial cells, and from reduction mammoplasty, benign breast and breast tumor fibroblast cells.

[4]   Formation of conglomerates from HBE-Cl, MCF10-2F and MCF10-3F, along with aggregates of the ER+ E2-responsive MCF7 human breast cancer cell line; conglomerates being defined as three-deimensional structures formed in response to a shift from a low to routine calcium environment and resembling human breast parenchymal organization *in vivo*. Detailed morphologic characterization as well as analysis for ER expression are in progress.

[5]   Experiments examining for ER expression and for E2 growth responsiveness of HBE-CL to date have not revealed E2 responsive growth or reliable ER expression, in contrast to MCF7 controls evaluated simultaneously that were E2 growth responsive and expressed ER.

In summary, although Dr. Soule's death caused a change in important staff, substantial progress, somewhat delayed from the projected "Statement of Work", has been accomplished in year 1. We anticipate making up this delay in the remaining two years of funding.

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